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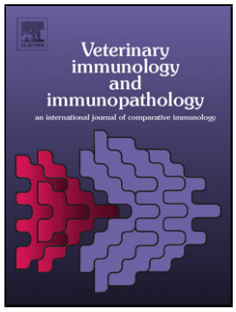

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Neospora caninum truncated recombinant proteins formulated with liposomes and CpG-ODNs triggered a humoral immune response in cattle after immunisation and challenge

Running head: Humoral immunity against recombinant proteins of *N. caninum* formulated with liposomes and CpG-ODNs in cattle

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Highligths

- Truncated proteins rMIC1, rMIC3, rSRS2 and rGRA7 were immunogenic in cattle
- The recombinant protein vaccine stimulated specific IgG, IgG1 and IgG2 in cattle
- Specific IgG, IgG1 and IgG2 increased in vaccinated steers after challenge
- Vaccinated steers had higher antibody levels than non-vaccinated ones after challenge

Abstract

Abortions caused by *Neospora caninum* are a serious problem in cattle production and require effective immunoprophylaxis. The objective of this work was to assess the humoral immune response to four recombinant (r) *N. caninum* antigens in cattle after immunisation and challenge. MIC1 and MIC3 proteins from the micronemes, SRS2 from the surface of tachyzoites, and GRA7 from the dense granules were expressed as truncated recombinant proteins in *Escherichia coli*. Cationic liposomes (Lip) and CpG oligodeoxynucleotides (CpG-ODNs) were used as adjuvant. Steers were assigned to three groups of six steers each and were inoculated twice subcutaneously, 21 days apart. The rP+Lip+CpG-ODN group received the truncated recombinant proteins rMIC1, rMIC3, rSRS2 and rGRA7 formulated with the adjuvant; the Lip+CpG-ODN group received the adjuvant alone; and the PBS group received sterile phosphate-buffered saline. All steers were subcutaneously challenged with the NC-1 strain of *N. caninum* 35 days after the second dose of immunisation. Steers from the rP+Lip+CpG-ODN group developed specific IgG, IgG1 and IgG2 against the four recombinant proteins after immunisation. After challenge, IgG against rMIC1 and rMIC3 was detected in rP+Lip+CpG-ODN group and against rSRS2 and rGRA7 in all groups. IgG1 and IgG2 against the four recombinant proteins remained high after challenge in the rP+Lip+CpG-ODN group. Indirect ELISA detected anti-*N. caninum* antibodies after challenge in all groups, with the highest level of antibodies being detected in the rP+Lip+CpG-ODN group. The recombinant vaccine formulated with rMIC1, rMIC3, rSRS2 and rGRA7 using Lip+CpG-ODN as adjuvant was immunogenic in cattle and the humoral immune response after challenge was enhanced in vaccinated cattle.

Keywords: Vaccine, *Neospora caninum*, Recombinant proteins, Liposomes, CpG oligodeoxynucleotides, Cattle

1. Introduction

Neospora caninum is an intracellular apicomplexan parasite that can cause abortion in cows (Thilsted and Dubey, 1989) or birth of infected calves with neuromuscular damage (Dubey et al., 1992). *N. caninum* infection causes significant economic losses in cattle production worldwide (Reichel et al., 2013). *N. caninum* can be transmitted by oocysts that canids shed through their faeces, contaminating water and fodder. In addition, the parasite can be transmitted transplacentally from the infected dam to the foetus (Anderson et al., 1997). Strategies to interrupt the life cycle of the parasite and, consequently, control the disease include farm biosecurity and culling of infected animals (Dubey et al., 2007). The wildlife involved in the life cycle of *N. caninum* includes rodents, birds and many mammals that are difficult to control (Almería, 2013), and culling of infected cows is not always economically feasible. Thus, additional control measures such as treatment or vaccination are needed.

Experimental live vaccines based on naturally attenuated *N. caninum* isolates showed to protect cattle completely (Williams et al., 2007) or partially (Rojo-Montejo et al., 2013) against abortion after experimental infection during pregnancy. However, live vaccines have several disadvantages associated with the cost, time and labour of *in vitro* parasite culture, the limited shelf life of the vaccine, latency in the intermediate host and the potential for reversion to pathogenicity (Reichel et al., 2015). These disadvantages promote the development of subunit-based vaccines. Recombinant protein vaccines have advantages, such as safety, standardised composition, and simplified production and distribution (Nishikawa, 2017).

The *N. caninum* recombinant antigens MIC1 and MIC3 from micronemes, GRA7 from the dense granules and SRS2 (SAG1-related sequence) from the surface of tachyzoites conferred protection against acute infection as well as cerebral infection in adult mice, and increased offspring survival in pregnant mice after challenge with tachyzoites of NC-1 strain (Alaeddine et al., 2005; Cannas et al., 2003; Nishikawa et al., 2009; Pinitkiatisakul et al., 2007). Therefore, they are considered promising vaccine candidates (Ellis et al., 2008). Recombinant protein vaccines were also evaluated in calves (Nishimura et al., 2013) and pregnant heifers (Hecker et al., 2014). The combination of multiple antigens in recombinant vaccines may generate an immune response against molecules involved in different stages of the parasite pathogenesis, helping to avoid or eliminate the infection in a more effective way.

The use of a proper adjuvant is a key factor in the design of subunit vaccines, since adjuvants are able to direct the immune response profile and increase the vaccine efficacy (Monney and Hemphill, 2014). An effective immune response for the control of *N. caninum* infection requires an active Th1 cellular immune response with production of a specific IgG2 to control intracellular parasites as well as enhance the lysis of parasites before cell invasion (Innes et al., 2002, Almería et al, 2009). Liposomes (Lip) can mimic pathogenic microorganisms carrying large amounts of antigens and immunostimulatory agents such as oligodeoxynucleotides with unmethylated CpG sequences (CpG-ODN) (Frézard, 1999). In previous works in mouse models, the combination of liposomes with CpG-ODNs (Lip+CpG-ODN) stimulated powerful immune responses with a Th1 profile (Kim et al., 2011; Reidel et al., 2017). In bovine models, CpG-ODNs were biologically capable of stimulating both innate and adaptive immune responses (Ioannou et al., 2002; Nichani et al., 2004; Pontarollo et al., 2002; Wedlock et al., 2005). In a more recent work, immunisation of calves with two

recombinant proteins from *Staphylococcus aureus* formulated with Lip+CpG-ODN induced a strong humoral immune response, with production of specific IgG2 and memory immune response (Reidel et al., 2019).

The aim of this work was to develop a recombinant protein vaccine formulated with the antigens MIC1, MIC3, GRA7 and SRS2 from *N. caninum* expressed as recombinant proteins and using Lip+CpG-ODN as adjuvant, and to evaluate the humoral immune response elicited in cattle after vaccination and challenge with live *N. caninum* tachyzoites of NC-1 strain.

2. Materials and methods

2.1. Parasites

Neospora caninum tachyzoites of the NC-1 strain (Dubey et al., 2002) were maintained *in vitro* by continuous passage in Vero (African green monkey kidney) cell culture. After harvest and purification as previously described (Novoa et al., 2020), tachyzoite viability and number were determined by trypan blue dye exclusion test followed by counting in a Neubauer chamber. For the challenge inoculum, the concentration was adjusted to 5×10^7 tachyzoites/ml in phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4).

2.2. *In silico* protein sequence analysis

The prediction algorithm SignaIP 5.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptides (Bendtsen et al., 2004). TMpred algorithm was used to predict the transmembrane helixes of each protein (http://embnet.vital-it.ch/software/TMPRED_form.html) (Hofmann and Stoffel, 1993).

2.3. Genetic construction

Genomic DNA (gDNA) and total RNA were purified from *N. caninum* tachyzoites by phenol–chloroform–isoamyl alcohol and acid phenol-guanidinium thiocyanate-chloroform (TRIzol™ reagent, Invitrogen, Waltham, MA, USA) extraction methods, respectively.

N. caninum MIC1, MIC3, SRS2, and GRA7 proteins were expressed as truncated recombinant (r) proteins, without signal peptides and transmembrane domains. gDNA was used as a template for coding DNA (cDNA) amplification by PCR of rSRS2 and rGRA7 proteins. Total RNA was employed as a template for single-stranded DNA synthesis of MIC1 and MIC3 genes (genes with introns) using specific forward primers (Table 1) and Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). Single-stranded DNA was used as template for cDNA amplification by PCR of rMIC1 and rMIC3 proteins. PCRs were performed using Platinum® Pfx DNA Polymerase (Invitrogen) and specific primers (Table 1). Amplicons were cloned into pGEM-T Easy vector (Promega) and subsequently subcloned into pET24a (Novagen, MiliporeSigma, Darmstadt, Germany) using BamHI and EcoRI restriction enzymes. The recombinant plasmids, pMIC1, pMIC3, pSRS2 and pGRA7, were confirmed by DNA sequencing (Biotechnology Institute, CICVyA, INTA, Argentina) and used to transform *E. coli* BL21 RIL (DE3) pLysS competent cells (Novagen).

2.4. Protein expression and purification

E. coli BL21 RIL (DE3) pLysS competent cells carrying the plasmids pMIC1, pMIC3, pSRS2 or pGRA7 were cultured at 37 °C in 500 mL of lysogeny broth medium, supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol to an optical density at 600 nm (OD_{600nm}) = 1. Protein expression was induced with 1% lactose. After 3 h of incubation at 37 °C, bacteria were harvested by centrifugation and suspended in 10 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) containing 1:1,000 of protease inhibitor cocktail set III (Calbiochem, MilliporeSigma). The cell suspension was

lysed by two passes through a cell disruptor at 20,000 psi (Emulsiflex B15; Avestin, Ottawa, Ontario, Canada). Soluble and insoluble fractions were separated by centrifugation (35,000 × *g*, 4 °C, 30 min).

rMIC3 and rGRA7 proteins were purified from their respective soluble fraction using Ni^{+2}-NTA agarose (Qiagen, Hilden, Germany), following a previously described protocol for purification of soluble recombinant proteins (Novoa et al., 2020). Finally, the buffer was exchanged into buffer A (25 mM Na_2HPO_4, 150 mM NaCl, pH 8) by dialysis at 4 °C overnight (ON). rMIC1 and rSRS2 proteins were purified under denaturing conditions from the insoluble fraction following a previously described protocol for purification of recombinant proteins from inclusion bodies (Sarli et al., 2020). The proteins were eluted in denaturing buffer (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM β-mercaptoethanol, 6 M urea, pH 8) and then diluted with buffer A at a concentration of 1 µg/µl. Finally, the denaturing buffer was exchanged completely into buffer A by ON dialysis at 4 °C.

The purity of the proteins was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The molar concentration in pure samples was calculated using the absorbance at 280 nm and the theoretical molar extinction coefficient (ε_{280nm}) for each protein (27,680 M^{-1} cm^{-1}, 20,535 M^{-1} cm^{-1}, 19,075 M^{-1} $cm^{-}1$, and 11,460 M^{-1} cm^{-1} for rMIC1, rMIC3, rSRS2 and rGRA7, respectively). Proteins were stored at 4 °C until use.

2.5. Vaccine formulation

The experimental immunogen consisted of 100 µg/dose of each of the recombinant *N. caninum* antigens, rMIC1, rMIC3, rSRS2 and rGRA7, equivalent to 400 µg total recombinant protein (rP)/dose. The vaccine was formulated with Lip+CpG-ODN as adjuvant (rP+Lip+CpG-ODN). This adjuvant was developed and produced in the Experimental Immunology Laboratory of the Faculty of Biochemistry and Biological Sciences,

Universidad Nacional del Litoral, Santa Fe, Argentina. Briefly, liposomes were prepared using dipalmitoylphosphatidylcholine, cholesterol and stearylamine in a 7:2:2 mol/mol ratio and 4 mM as final lipid concentration via the ethanol injection method (Wagner et al., 2006). CpG-ODNs were used at a 7.5 nmol/mL concentration. The CpG-ODN sequence used in this study was a 23-mer 5′-tcgtcgtttgtcgttttgtcgtt-3′ (Mulongo et al., 2013), synthesized with phosphodiester (PO) bonds (Invitrogen).

2.6. Animals and treatments

Eighteen 3-y-old Holstein steers belonging to the dairy herd of INTA Rafaela Experimental Station (Rafaela, Santa Fe, Argentina) were selected. Before the beginning of the trial, the presence of anti-*N caninum* antibodies was evaluated in sera from all steers, at three different times, using the indirect fluorescent antibody test (IFAT) (Paré et al., 1995) and a competitive inhibition ELISA (ciELISA) (Novoa et al., 2020). Animals were randomly assigned to three experimental groups of six steers each: rP+Lip+CpG-ODN group received the recombinant protein vaccine; Lip+CpG-ODN group received the adjuvant alone, and PBS group received sterile phosphate-buffered saline. Steers were inoculated subcutaneously (sc) in the sub-scapular region with two doses of 2 ml of the designated inoculum, administered 21 days apart. Local inflammatory reactions at the injection site were evaluated daily for one week after each inoculation. Thirty-five days after the second dose, all steers were challenged sc with 1×10^8 *N. caninum* tachyzoites of NC-1 strain. An aliquot of the parasite inoculum was seeded in Vero cells to confirm parasite viability. Animals were bled by puncture of the coccygeal vein, weekly during 14 weeks, starting 1 week before the first dose. Blood was allowed to clot and sera were collected via centrifugation and stored at −20 °C until processing.

All the procedures were carried out according to the guide for the care and use of agricultural animals in research and teaching (Federation of Animal Science Societies; FASS, 2010), and approved by the Committee for the care and use of experimental animals of the Santa Fe Regional Center (CICUAE), from the National Institute of Agropecuary Technology, Argentina (license 18-009).

2.7. Serological determinations

2.7.1. Enzyme-Linked ImmunoSorbent Assays

The antibody responses (IgG, IgG1 and IgG2) to rMIC1, rMIC3, rSRS2 and rGRA7 proteins were determined by indirect ELISA (iELISA). Optimal dilutions of sera, antigens and conjugates were established using checkerboard titrations (Crowther, 2000). Briefly, 96-well ELISA plates (Nunc; Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 µL of each recombinant protein (30 µg/mL) diluted in PBS, and incubated ON at 4 °C. The coated plates were first incubated with 10% (w/v) skimmed milk powder (Svelty, Nestlé, Buenos Aires, Argentina) dissolved in PBS and then with serum samples diluted 1:4 or 1:10 for detection of antibodies against rMIC1 and rGRA7 or rMIC3 and rSRS2, respectively. All samples were assayed in duplicate. Then, the plates were incubated with horseradish peroxidase-conjugated anti-bovine IgG, anti-bovine IgG1 mouse antibodies (MiliporeSigma), or non-conjugated anti-bovine IgG2 mouse antibody (MiliporeSigma), followed by incubation with anti-mouse IgG peroxidase-conjugated rabbit antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA). Sera and antibodies were diluted in 10% (w/v) skimmed milk powder dissolved in PBS with 0.1% (v/v) of Tween 20. All incubations were carried out at 25 °C for 1 h. Between steps, plates were washed three times with 0.1% (v/v) Tween 20 in PBS. Lastly, 100 µL of 1 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (MilliporeSigma) and 0.03% v/v of H_2O_2 in 0.05 M

sodium citrate buffer (pH 4.5) were added. Optical density (OD) was measured at 405 nm using an ELISA plate reader (Labsystems Multiskan FC; Microlat, Buenos Aires, Argentina). Specific anti-*N. caninum* antibodies were detected using an iELISA based on a *N. caninum*-tachyzoite lysate ($iELISA_{tach}$) and a ciELISA based on a recombinant truncated variant of the major surface antigen from *N. caninum* tachyzoites ($ciELISA_{tSAG1}$) performed as described previously (Moore et al., 2011; Novoa et al., 2020). The results were expressed as percentage of positivity (%P) for $iELISA_{tach}$ and percentage of inhibition (%I) for $ciELISA_{tSAG1}$.

2.7.2. Immunoblot

Specific anti-*N. caninum* antibodies were detected by immunoblot (IB) as described previously (Campero et al., 2015). Control sera and sera from steers previous to the challenge (day 28) were diluted 1:100 in tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% v/v of Tween-20 and 5% w/v of skimmed milk (TBS-T-M). Anti-bovine IgG peroxidase conjugate produced in rabbit (1:1,000 in TBS-T-M) (MilliporeSigma) was used. The antigen-antibody reaction was revealed with the colorimetric substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (MilliporeSigma) and 0.1% v/v of hydrogen peroxide (H_2O_2).

2.8. Statistical analysis

The levels of IgG, IgG1 and IgG2 antibodies against recombinant proteins and levels of IgG against *N. caninum* tachyzoite lysate and SAG1 protein were compared between groups using a Generalized Linear Model with Gamma distribution as a link function, considering the frequency distribution of the response variable. Repeated measurements were used in the analyses of IgG levels. All statistical analyses were considered significant at $p < 0.05$. Data were analysed using the software InfoStat from Universidad Nacional de Córdoba, Córdoba, Argentina (Di Rienzo et al., 2011).

3. Results

3.1. Sequences analysis, and expression and purification of recombinant proteins

The protein fragments predicted to be exposed on the outer face of the parasite organelle or tachyzoites membrane by the *in silico* analysis were selected for expression (Table 2). rMIC3 and rGRA7 proteins were expressed in soluble form in the *E. coli* cytoplasm with an average yield, after purification, of 3 and 10 mg per litre of culture medium, respectively. rMIC1 and rSRS2 proteins were expressed in inclusion bodies; after their purification under denaturing conditions and refolding, an average yield of 3 and 5 mg per litter of culture medium, respectively, was obtained. After electrophoretic separation, rMIC1, rMIC3 and rSRS2 purified proteins presented a thick band of the expected molecular weight (MW) for the monomeric form of the protein and a thin band of the expected MW for the dimeric form of the protein. rGRA7 migrated as a thick band of the expected MW.

3.2. Local inflammatory reactions

No inflammatory reactions were observed at the inoculation site in any steer.

3.3. Humoral immune response

3.3.1 IgG against rMIC1, rMIC3, rGRA7 and rSRS2 in serum

Immunoglobulin G levels against rMIC1, rMIC3, rGRA7 or rSRS2 in serum samples were assessed (Figure 2-A). Before inoculations, no antibodies against the four recombinant proteins or against *N. caninum* tachyzoite lysate were detected in sera from the steers included in the study.

Regarding the rP+Lip+CpG-ODN formulation before challenge, steers in this group maintained significantly higher levels of antibodies against rMIC1, rMIC3 and rSRS2 than Lip+CpG-ODN and PBS groups from 14 days after the first dose and against rGRA7 from 7 days after the second dose administration until the end of the trial. The highest IgG levels

anti-rMIC1, anti-rMIC3 and anti-rGRA7 were detected 7 days after the second dose (day 28), whereas IgG anti-rSRS2 reached the maximum level 21 days after the second dose (day 42). After the peak, IgG levels against rMIC1, rMIC3 and rSRS2 decreased progressively. IgG levels against rGRA7 remained high until the end of the trial (day 84).

After challenge, an increase in IgG levels against rMIC1 and rMIC3 was observed only in rP+Lip+CpG-ODN group. On the other hand, the three evaluated groups presented an increase of IgG anti-rSRS2 and anti-rGRA7 14 days after challenge. Nevertheless, anti-rSRS2 and anti-rGRA7 levels 21 days after challenge were higher in rP+Lip+CpG-ODN group than in Lip+CpG-ODN and PBS groups.

3.3.2 IgG against-*N. caninum* antibodies in serum

According to the iELISA$_{tach}$, steers from rP+Lip+CpG-ODN group showed median levels of anti-*N. caninum* antibodies above the cutoff at days 35 and 49 before challenge, and from day 63 (7 days after the challenge) to the end of the trial. Before challenge, noanti-*N. caninum* antibodies were detected in steers from Lip+CpG-ODN and PBS groups. Moreover, sera of steers from rP+Lip+CpG-ODN group revealed by IB a different pattern of bands than sera of steers from control groups (Figure 3). After challenge, an increase of anti-*N. caninum* antibody level above the cutoff was detected in serum samples of steers from Lip+CpG-ODN and PBS groups (Figure 2-B). The maximum %P was measured 14 days after challenge (day 70) in the three experimental groups; nevertheless, the rP+Lip+CpG-ODN group presented higher %P than Lip+CpG-ODN and PBS groups from day 70 to the end of the trial (day 84). At that time, the pattern of bands revealed by IB was similar for steers from all groups (data not shown).

Before challenge, no anti-*N. caninum* antibodies were detected in any steer by ciELISA$_{tSAG1}$. Fourteen days after challenge, anti-*N. caninum* antibody levels above the cutoff were

detected by the ciELISA$_{tSAG1}$ in all steers from the three experimental groups (Figure 2-C). According to this assay, rP+Lip+CpG-ODN group presented no differences in %I from the control groups.

3.3.3 IgG1 and IgG2 against rMIC1, rMIC3, rGRA7 and rSRS2 in serum

Levels of IgG1 and IgG2 specific for each protein were determined (Figure 4) 7 days after the second dose (day 28) and 15 days after challenge (day 70).

Regarding IgG1 production, the rP+Lip+CpG-ODN formulation was able to increase the specific antibody levels against the four recombinant proteins at both evaluated times, days 28 and 70. Moreover, in all cases, steers from rP+Lip+CpG-ODN group had significantly higher IgG1 levels than steers from Lip+CpG-ODN and PBS groups.

Concerning the production of specific IgG2, steers from rP+Lip+CpG-ODN group presented higher levels of anti-rMIC1, anti-rMIC3 and anti-rGRA7 antibodies than steers from Lip+CpG-ODN and PBS groups after the second dose (day 28). Even though steers from rP+Lip+CpG-ODN group maintained high IgG2 levels against these three recombinant proteins after challenge, no significant differences from the control groups were detected at day 70. IgG2 anti-rSRS2 reached higher levels than control groups only after challenge.

4. Discussion

The inoculation of live naturally attenuated *N. caninum* parasites before gestation was found to protect pregnant dams against abortion when challenged with a pathogenic strain of *N. caninum* (Rojo-Montejo et al., 2013; Weber et al., 2013; Williams et al., 2007). Since there is a potential risk in live vaccines, experimentation to find an alternative vaccine continues. We demonstrated that four *N. caninum* recombinant antigens (rMIC1, rMIC3, rSRS2 and rGRA7) formulated with liposomes and using CpG-ODNs as immunostimulant were

immunogenic in a non-pregnant cattle model. Vaccine efficacy to control *N. caninum* infection should be evaluated in pregnant cows because abortion is the main clinical sign of neosporosis (Monney and Hemphill, 2014); however, the non-pregnant cattle model is more ethical and allows us to determine and characterize the immune response generated by the experimental immunogen (Nishimura et al., 2013). It is known that males and females differ in their immune responses against antigens (Klein and Flanagan, 2016), and it was shown that females generate greater antibody responses to both live and inactivated vaccines when compared to males (Fink and Klein, 2018). Therefore, the castrated male bovine model that we used, could underestimate the humoral immune response stimulated by the four recombinant proteins formulated with Lip+CpG-ODNs in cattle, but could be used as a first estimation of the humoral immunity stimulated by the vaccine.

Immunisation of mice with recombinant MIC1 (Alaeddine et al., 2005), MIC3 (Cannas et al., 2003), both MIC1 and MIC3 combined with ROP2 (Debache et al., 2009), GRA7 (Nishikawa et al., 2009) or SRS2 (Pinitkiatisakul et al., 2007) proteins resulted in a significant reduction of brain infection in adult mice or higher survival of offspring in pregnant mice after challenge with *N. caninum* NC-1 strain. Recombinant GRA7 protein entrapped in oligomannose-coated liposomes tested in calves induced protective immune response against the challenge with *N. caninum* NC-1 strain, evidenced by the lower number of parasites found in the brains of vaccinated calves than in the control group (Nishimura et al., 2013). In our work, a recombinant vaccine formulated with rMIC1, rMIC3, rSRS2 and rGRA7 proteins adjuvanted with liposomes and CpG-ODN, stimulated the production of specific antibodies against the four proteins in cattle.

After challenge with *N. caninum* NC-1 strain, there was an increase in IgG levels against rMIC1 and rMIC3 proteins in vaccinated steers, but not in steers from control groups. MIC1 and MIC3 proteins are involved in parasite adhesion to the host cell and parasite motility. These proteins are discharged from micronemes after apical attachment of the parasite to the host cell surface (Buxton et al., 2002). For this reason, MIC proteins are present in the extracellular environment at a low concentration and this could be the cause of the absence of detectable antibodies after the first contact of cattle with the parasite. Conversely, cattle previously immunised with rMIC1 and rMIC3 proteins showed a detectable increase of anti-rMIC1 and -rMIC3 antibodies after challenge. The low concentration of these proteins in the extracellular environment after the experimental infection might not be enough to stimulate a detectable primary immune response through the measurement of antibodies, but would be enough for a secondary response. Antibodies against rSRS2 and rGRA7 proteins were detected after challenge in serum from steers of the three experimental groups. SRS2 is present in the surface of the parasites and is involved in the initial contact with the host cell (Buxton et al., 2002). GRA7 proteins are localized in dense granules and released into the parasitophorous vacuole. Following host cell lysis due to parasite multiplication, large amounts of this protein are released to the extracellular environment (Buxton et al., 2002). Both proteins are useful antigens for the diagnosis of *N. caninum* infection in cattle (Aguado-Martínez et al., 2008; Álvarez-García et al., 2006; Borsuk et al., 2011; Schares et al., 2000). Therefore, the difference observed between groups in the production of antibodies against the four proteins after challenge could be related to localization of proteins in the tachyzoites, their abundance in the extracellular environment after infection with *N. caninum* and the previous activation of the immune system.

Even though the IgG levels against rGRA7 were higher in the steers from rP+Lip+CpG-ODN group than in steers from control groups only after the application of the second vaccine dose, the difference between groups was significant until the end of the trial. This finding demonstrates a low but clear immunogenicity of this recombinant antigen. The rGRA7 fragment expressed in this work (residues 29-138) differs from the fragments evaluated as immunogen in previous works, which used the full-length protein lacking the signal peptide (residues 29 to 217) (Hecker et al., 2014; Nishimura et al., 2013). In a previous study, sera from naturally and experimentally infected cattle recognized a truncated GRA7 recombinant protein that included residues 27 to 217, but did not recognize other minor fragments of the protein (Abdelbaky et al., 2018). Therefore, in our work, the lack of the carboxyl-terminal fragment might be the cause of the reduced immunogenicity of rGRA7 protein in cattle.

After immunisation, the iELISA$_{tach}$ detected an increase of anti-*N. caninum* antibodies in sera of steers from rP+Lip+CpG-ODN group. The IB performed using a tachyzoite lysate as antigen revealed antigen-antibody reaction when vaccinated steers sera was used. This observation indicates that antibodies generated in response to vaccination with recombinant proteins could recognize the native forms of the proteins present in *N. caninum* tachyzoites. The bands observed were of approximately of 20, 35, 40, 55 and 60 kDa and the MW previously reported for GRA7, MIC1, MIC3 and SRS2 proteins were 17 kDa (Alvarez-Garcia et al., 2007), 60 kDa (Keller et al., 2002), 38 kDa (Sonda et al., 2000) and 41 kDa (Nishikawa et al., 2001), respectively.

After challenge, the iELISA$_{tach}$ detected an increase of anti-*N. caninum* antibodies in sera of steers from all groups, but sera of vaccinated steers showed a higher %P than sera of steers from control groups. These results evidence that the fragments selected for expression as

recombinant proteins include immunogenic epitopes from the proteins naturally expressed in *N. caninum* tachyzoites. Therefore, we demonstrated that the selected fragments rMIC1, rMIC3, rSRS2 and rGRA7 are immunogenic in cattle. On the other hand, the ciELISA$_{tSAG1}$ did not detect differences between groups. These results indicate that the humoral immune response against SAG1 (protein not included in the vaccine) was similar in the three experimental groups, but the humoral immune response to the proteins included in the vaccine was enhanced in the vaccinated group.

The evaluated recombinant protein vaccine generated an increase in IgG1 and IgG2 levels against rMIC1, rMIC3, rSRS2 and rGRA7 in immunised steers before and after challenge. The stimulation of IgG2 is related to a Th1 immune response that corresponds with the natural infection immune profile (Staska et al., 2003) and could be protective against *N. caninum* abortion (Almería et al., 2009). The use of a proper adjuvant is a key factor in directing the immune response profile (Monney and Hemphill, 2014). In previous works performed using mice and calves as experimental models, the combination of cationic liposomes and CpG-ODN with phosphodiester (PO) bonds stimulated the humoral immune response with high antibody titres and production of IgG2a (Reidel et al., 2017, 2019). Moreover, the formulation generated memory immune response in the calves (Reidel et al., 2019). Oligomannose-coated liposomes were used as adjuvant in a vaccine against *N. caninum*, using GRA7 antigen. This vaccine was protective against the challenge with *N. caninum* NC-1 strain in calves (Nishimura et al., 2013). In our work, neither the memory immune response nor the protection against the experimental infection was evaluated, but higher IgG2 levels were observed against rMIC1, rMIC3, rSRS2 and rGRA7 proteins in vaccinated steers before challenge and were maintained high after challenge, suggesting a

Th1 profile induction. After challenge, in some of the non-vaccinated cattle, an increase of IgG2 levels was observed against rMIC1, rMIC3 and rGRA7. However, further analyses are required to establish the immune response profile (Th1 or Th2) induced by this vaccine.

Different adjuvants were used in the formulation of vaccines to prevent *N. caninum* infection and were tested in cattle, including immune stimulating complexes matrix (ISCOM-matrix) (Hecker et al., 2014; Moore et al., 2011), soy lecithin/β-glucan adjuvant (Mansilla et al., 2013) and oligomannose-coated liposomes (Nishimura et al., 2013). These vaccines generated a detectable humoral and cellular immune response; however, the protection against abortion was not confirmed. In adjuvant selection to design a vaccine, the required immune profile, the availability, the cost and the possible generation of adverse side effects should be considered. The PO CpG-ODNs, which we used in our vaccine formulation, have a lower production cost than phosphorothioate (PS) CpG-ODNs used in several studies (Ioannou et al., 2002; Nichani et al., 2004; Pontarollo et al., 2002; Wedlock et al., 2005). PS CpG-ODNs have a longer *in vivo* half-life than PO CpG-ODNs (Mutwiri et al., 2004), but they are associated with adverse side effects in mice and primates (Shargh et al., 2012). These are relevant factors to consider in vaccine production, commercialization, and consumer acceptance. Thus, in this first evaluation, rMIC1, rMIC3, rGRA7 and rSRS2 proteins from *N. caninum* using liposomes and CpG-ODN as adjuvant and immunostimulant in cattle showed to be immunogenic, generating a humoral immune response similar to that generated after the experimental infection. Nevertheless, further research is necessary to assess if the vaccine is protective against infection and abortion.

5. Conclusion

In the present work, we demonstrated that the truncated recombinant proteins rMIC1, rMIC3, rSRS2 and rGRA7 formulated with a liposomal adjuvant using PO CpG-ODN as immunostimulant were immunogenic in cattle. After challenge, an increase of IgG levels against rMIC1 and rMIC3 antigens was detected only in the vaccinated group and against rSRS2 and GRA7 antigens in steers from three experimental groups. The $iELISA_{tach}$ based on a tachyzoite lysate detected an increase of anti-*N. caninum* antibodies after challenge in the three experimental groups, with a higher level of antibodies detected in the vaccinated group. The IB detected that sera from vaccinated steers after immunisation reacted with proteins present in a tachyzoite lysate. This result demonstrates that the fragments of the proteins selected for expression as recombinant proteins include immunogenic epitopes. The vaccine elicited a humoral immune response against rMIC1, rMIC3, rSRS2 and rGRA7 proteins with an increase in specific IgG1 and IgG2 levels that remained high after the challenge.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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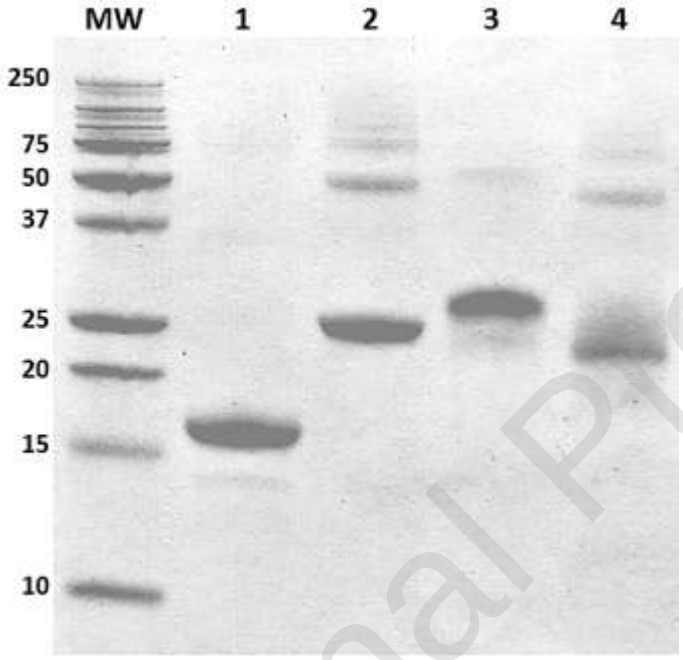

Figure 1: Sodium dodecyl sulfate-12 % polyacrylamide gel electrophoresis. Purified recombinant proteins from *Neospora caninum* stained with Coomassie Brilliant Blue R-250. MW: molecular weight marker (kDa); lane 1: rGRA7; lane 2: rMIC1; lane 3: rMIC3 and lane 4: rSRS2.

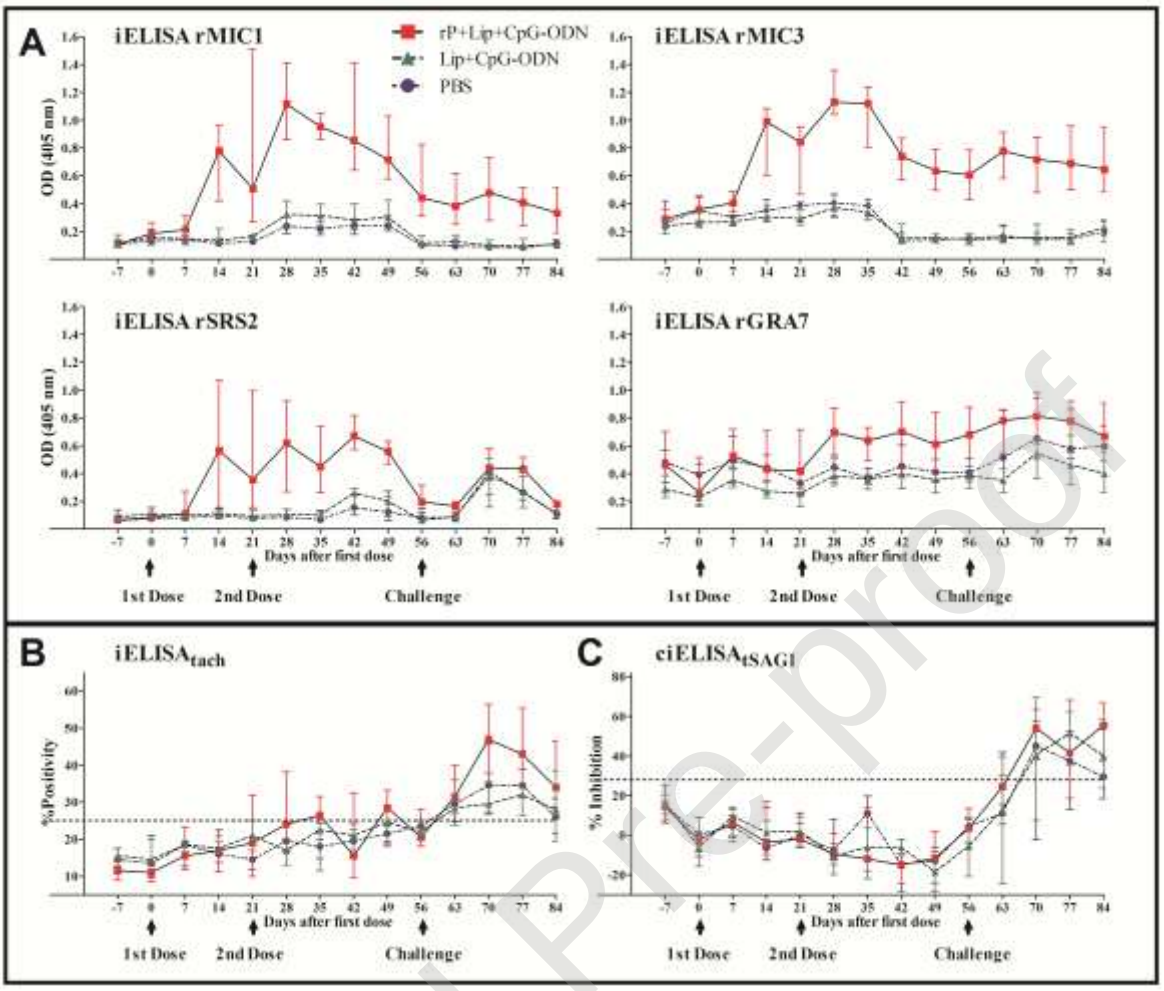

Figure 2: A- Specific IgG detection in sera of steers immunised with the recombinant protein vaccine (rP+Lip+CpG-ODN), the adjuvant (Lip+CpG-ODN) or buffer (PBS), by indirect ELISA. Sera were tested against single recombinant antigens. Optical density (OD) at 450 nm was measured. B- Specific IgG detection in sera against a lysate of *Neospora caninum* tachyzoites measured by indirect ELISA ($iELISA_{tach}$). Percentage of positivity (%P) is shown. C- Specific IgG detection in sera against tSAG1 antigen from *N. caninum* measured by competitive inhibition ELISA ($ciELISA_{tSAG1}$). Percentage of inhibition (%I) is shown. A Generalized Linear Model with Gamma distribution of repeated measures was performed in

all cases. Median ± IQR of six animal sera from each group is shown. Horizontal dotted lines indicate the cutoff point. Arrows indicate immunisation and challenge times.

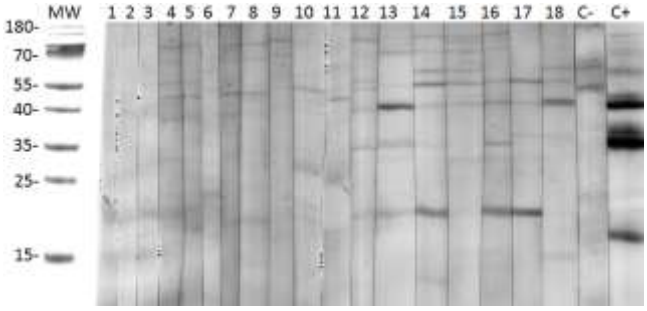

Figure 3: Immunoblot analysis using a *Neospora caninum* tachyzoite lysate as antigen and sera of steers after immunisation. Strips 1 to 6 were incubated with sera from steers inoculated with PBS; strips 7 to 12 were incubated with sera from steers inoculated with the adjuvant (Lip+CpG-ODN) and strips 13 to 18 were incubated with sera from steers immunised with the vaccine (rP+Lip+CpG-ODN). MW = molecular weight marker PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific). C+: Sera from a cow experimentally infected with *N. caninum*. C-: Sera from an uninfected cow.

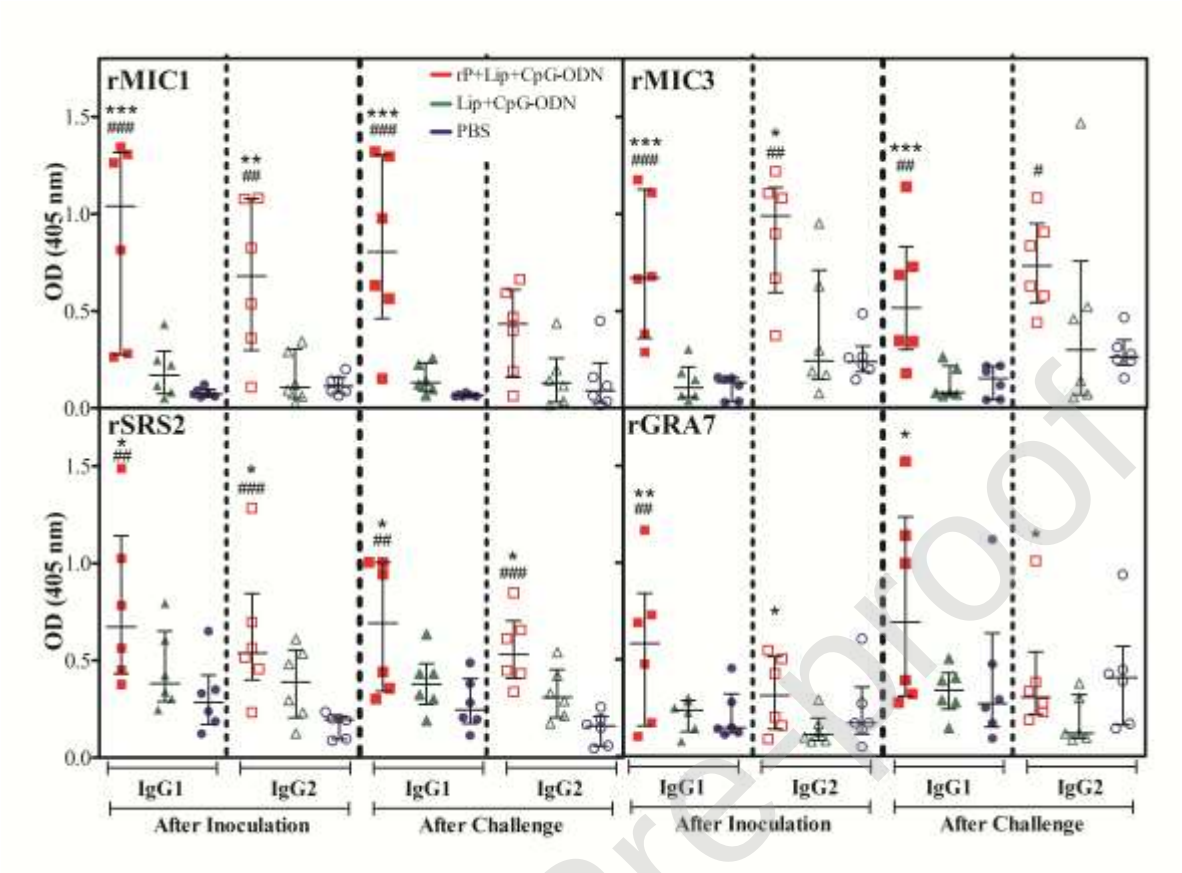

Figure 4: Specific IgG1 and IgG2 detection against rMIC1, rMIC3, rSRS2 and rGRA7 recombinant proteins in sera of steers immunised with the recombinant protein vaccine (rP+Lip+CpG-ODN), the adjuvant (Lip+CpG-ODN) or buffer (PBS), measured by indirect ELISA 7 days after the second dose (day 28) and 14 days after challenge (day 70). Sera were tested against single recombinant antigens. Optical density (OD) at 450 nm was measured. A Generalized Linear Model with Gamma distribution was performed. Median ± IQR of sera of six animals from each group is shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ of rP+Lip+CpG-ODN compared with Lip+CpG-ODN group and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ of rP+Lip+CpG-ODN compared with PBS group.

Table 1: Primers used for cDNA amplification of rMIC1, rMIC3, rGRA7 and rSRS2 proteins from *Neospora caninum*.

Gene	GenBank Access number	Forward primer	Reverse primer
MIC1	AF421187.1	ggatcc**atg**aaaacatacggagaagcgtcg	gaattc**tta***gtggtggtggtggtggtg*cttgtaacatctccattt
MIC3	XM_003880575.1	ggatcc**atg**gataaaagctacggtggtgactgc	gaattc**tca***gtggtgatgatggtgatg*actcgcggacaaactg
GRA7	JQ410455.1	ggatcc**atg**gacttagcaaccgaa	gaattc**tca***gtggtggtgatggtgatg*agtcttgacacgttt
SRS2	JQ410454.1	ggatcc**atg**ccattcaagtcggaaaatg	gaattc**tca***gtggtggtgatggtgatg*gcgttctttacattcgtc

Sequences are shown 5´to 3´. BamHI and EcoRI restriction sites are underlined in forward and reverse primers, respectively. The six-histidine tag sequence is shown in *italics*. Initiation and stop codons are shown in **bold letters,** in forward and reverse primers, respectively.

Table 2: Characteristics predicted for the full-length proteins MIC1, MIC3, GRA7 and SRS2 from *N. caninum* and their fragments selected for expression in *Escherichia coli* as truncated recombinant proteins.

Full-length proteins								Truncated recombinant proteins		
Protein name	Full length	Signal peptide	Cleavage site	Transmembrane regions				Name	Amino acids	Theoretical MW (kDa)
				i-o	o-i	i-o	o-i			
MIC1	1-459	1-18	18-20	1-19	181-198	222-240	392-409	rMIC1	20-179	23.9
MIC3	1-362	1-24	24-26	3-20	50-70	117-139		rMIC3	140-350	24.8
GRA7	1-217	1-26	26-28	4-21	139-157			rGRA7	29-138	13.9
SRS2	1-401	1-52	52-54	35-54	243-271	384-401		rSRS2	54-242	21.5

i-o: inside to outside transmembrane region. o-i: outside to inside transmembrane region